Seed Sprouts: the State of Microbiological Safety

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Mung bean sprouts have been propagated and used as food by the Chinese for almost 5,000 years (132). Presently, a wide variety of seed sprouts are grown at commercial establishments or in the home for consumption either raw or lightly cooked. In the United States, mung bean, alfalfa, clover, radish, and broccoli are among the most popular sprouts. Sprouts are propagated primarily by placing the seeds in trays, rotary drums, or bins and watering frequently for 4 to 7 days. Soil and soil-less planting mixes are used for only a few types of sprouts, including sunflower and wheatgrass, which are sometimes grown in greenhouses. Due to limited shelf life after harvest, packaged sprouts are distributed either locally or regionally.

Sprouts are believed by many consumers to be a healthy natural food. The health benefits of sprout consumption, in addition to the nutritional value of sprouts, are being confirmed by recent research efforts in several laboratories around the world. For example, the consumption of broccoli sprouts may provide chemoprotection against certain carcinogens as well as reduce the risk of developing hypertension and atherosclerosis (35, 190). Among the numerous tested vegetables, alfalfa sprouts ranked near the top in antioxidant activity (21).

Unfortunately, in the past decade, consumption of raw or lightly cooked seed sprouts contaminated with *Salmonella enterica* or *Escherichia coli* O157:H7 or O157:NM has been responsible for at least 27 outbreaks of infection in the United States, resulting in over 1,600 reported cases of

foodborne illness (54). During the period from 1973 through 1997, contaminated seed sprouts were responsible for 7 of 30 recorded U.S. outbreaks of foodborne salmonellosis linked to contaminated produce. Furthermore, contaminated sprouts were responsible for more multistate foodborne outbreaks than any other single produce item (154). Due to the international nature of seed distribution systems, some of these outbreaks have been international in scope (135). Sprouts were designated as a special food safety problem by the National Advisory Committee on Microbiological Criteria for Food in 1999 (117) for two reasons: (i) low levels of human pathogens on sprout seeds multiply into high populations during the sprouting process due to favorable conditions of temperature, moisture, and nutrient availability, and (ii) seed sprouts are often consumed raw, with no killing step. Sprouts are referred to as a "potentially hazardous food" in the 2001 U.S. Food and Drug Administration (FDA) Food Code (51). The FDA has issued a number of consumer advisories informing the public about the risks associated with eating raw sprouts; one such advisory states that "persons in high risk categories (i.e., children, the elderly, and the immunocompromised) should not eat raw or lightly cooked sprouts" (52). In 1999 the FDA released two guidance documents for the sprouting industry describing methods for reducing microbial safety hazards associated with sprouts; these documents recommended the use of antimicrobial seed treatments and the testing of spent irrigation water for *Salmonella* spp. and *E. coli* O157:H7 (49). In 2000 a food safety training video intended for commercial sprout growers was released by the FDA in collaboration with the Food and Drug Branch of the California Department of Health Services (59). Despite these efforts, sprout-related outbreaks continue to occur in the United States on an annual basis. This has led to a recent plan to initiate rule making by the FDA in an attempt to reduce the number of sprout-related outbreaks of foodborne illness (55).

In this chapter, we discuss the native microflora found on sprout seeds and sprouts, the most promising antimicrobial interventions for sanitizing seeds and sprouts, and the detection technologies that have been studied. We also provide insights on the unique challenges and opportunities that seed sprouts afford from a food safety perspective and discuss strategies for reducing the risk of future sprout-related outbreaks of foodborne illness.

NATIVE MICROBES ASSOCIATED WITH SPROUTS

Through the use of traditional bacteriological methods, sprout seeds and seed sprouts are known to harbor high populations of a variety of indigenous microbes. Populations of total mesophilic aerobes on seeds destined for sprouting are often in the range of 4 to 5 $\log_{10}$ CFU/g, with coliforms com-

prising 10% or less of the population (2, 3, 134, 158). Fecal coliforms are often detected, with populations of up to 3 $\log_{10}$ CFU/g reported (124, 134). The fecal coliforms cultured from sprout seeds are primarily *Klebsiella pneumoniae, Enterobacter aerogenes,* and *Enterobacter agglomerans* (95, 124, 158). Native bacteria can be internalized within seeds. Mundt and Hinkle (115) found that 13% of surface-sterilized alfalfa seeds and 15% of surface-sterilized soybean seeds harbored internalized bacteria. A wide variety of molds also can contaminate the surfaces of sprout seeds (3).

Sprout surfaces are populated with higher numbers of cultivable native microflora than sprout seeds (see below) and harbor some of the highest populations of indigenous microflora reported for nonspoiled fresh produce at retail. Favorable temperatures and frequent watering cycles used in sprout propagation, as well as leaching of nutrients from seeds and roots during sprout development, favor rapid growth of native microbes. Increases of 1 $\log_{10}$ CFU/g for yeast and mold and 3 to 5 $\log_{10}$ CFU/g for aerobic bacteria 2 days after initiation of germination have been reported (3, 23). Similar to those isolated from other types of produce, native bacteria isolated from sprouts consist primarily of gram-negative rods and appear to originate mainly from the seed (104, 146, 158, 172). Yeast and mold are also present (Fig. 1). Additional sources of microbes include the air (124), irrigation water, and workers.

Populations of aerobic mesophilic bacteria on seed sprouts range from 5 to 12 $\log_{10}$ CFU/g but are usually between 7 and 9 $\log_{10}$ CFU/g (3, 8, 134, 158, 177). Populations of aerobic mesophilic bacteria determined for laboratory-grown and commercially grown sprouts at retail are similar (39, 64, 127). Populations of *Enterobacteriaceae* in the range of 7 to 8 $\log_{10}$ CFU/g have been reported (39, 155), and populations of total coliforms, fecal coliforms, and yeast and mold vary widely (4 to 8, <1 to 6, and 2 to 6 $\log_{10}$ CFU/g, respectively) (3, 8, 9, 39, 127, 134, 146, 155, 156, 158, 172).

Enterobacteriaceae and *Pseudomonas* spp. are the primary cultivable indigenous bacteria on a variety of sprout types (8, 9, 128). The predominant bacterial genera on sprouts during the germination process can change with time. Mølbak et al. (110) reported that *Erwinia* and *Paenibacillus* spp. were dominant on 1-day-old alfalfa sprouts but were gradually replaced by *Pseudomonas,* which predominated at days 3 and 5. In Sweden and the United States, the most common coliform isolated from sprouts is *Pantoea agglomerans* (27, 95). In addition to *P. agglomerans,* other fecal coliforms that have been routinely isolated from sprouts include *K. pneumoniae, E. aerogenes, Enterobacter cloacae, Enterobacter sakazakii,* and *Citrobacter freundii* (8, 124, 127, 142, 155, 158). As fecal coliforms are often found on plant surfaces, including those of sprout seeds and sprouts, their presence is not a good

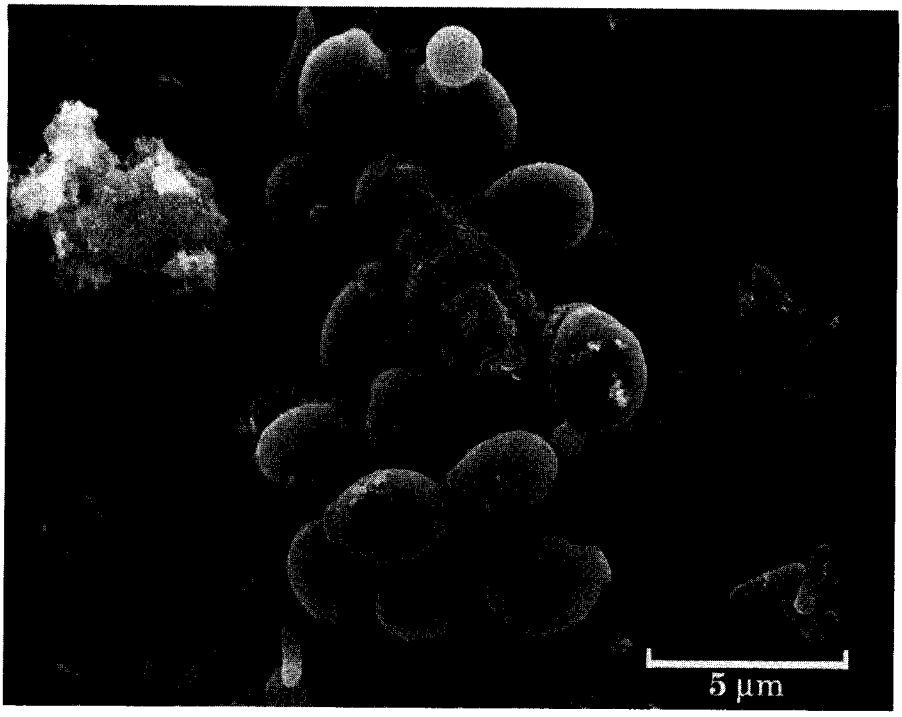

Figure 1 Scanning electron micrograph of native yeast and rod-shaped bacteria on the surface of a mung bean sprout cotyledon.

indicator of recent fecal contamination (127, 193). Representatives of the bacterial genera *Achromobacter, Acinetobacter, Alcaligenes, Bacillus, Chromobacterium, Flavobacterium, Hafnia, Moraxella, Lactococcus, Lactobacillus, Leuconostoc, Paenibacillus, Rahnella, Ralstonia, Serratia, Sphingomonas,* and *Stenotrophomonas* have also been isolated from sprouts (8, 18, 104, 110, 120, 121, 127, 144, 177, 187). Lactic acid bacteria appear to normally comprise a minor component of the total microflora on sprouting seeds (8, 9, 127, 155).

There is a lack of studies using modern methods of microbial ecology to investigate the native microflora on sprout seeds and sprouts. Matos et al. (104) utilized community-level physiological profiling, a technique based on carbon source utilization patterns (70), to compare the native aerobic heterotrophic microbial communities associated with five sprout types. Based on this methodology, communities on alfalfa and clover sprouts are more similar to each other than to communities on sunflower, mung bean, and broccoli sprouts. Communities on the latter three sprout types are distinct from one another. Comparison of communities on alfalfa and clover sprouts grown from different seed lots or in different commercial facilities indicates that

sprout type is responsible for more variability among communities than either seed lot or growing facility.

To our knowledge, cultivation-independent molecular techniques have not yet been applied for identifying microorganisms associated with seed sprouts. A recent analysis of GenBank entries indicated that only approximately half (27 of 53) of the discernible major phyla within the domain *Bacteria* have cultivated representatives (145). Plant phyllosphere and rhizosphere microbial communities are found to be more complex when examined by cultivation-independent methods than when examined by cultivation-based methods (91, 192). Thus, it is expected that the resident microflora on sprouts is more diverse than presently recognized. This idea was recently supported by results of two independent studies (105, 110) which compared direct microscope bacterial counts with agar plate counts of bacteria isolated from alfalfa sprouts. In most instances, only approximately 2 to 10% of the native bacteria present were cultivable.

Native bacteria are firmly attached to sprout surfaces, as evidenced by the inability of water washes after harvest to reduce populations by more than about 1 $\log_{10}$ CFU/g (8, 27, 126). Native microorganisms have been demonstrated by conventional scanning electron microscopy to be present both as solitary cells and as members of biofilms on sprout cotyledons, hypocotyls, and root surfaces (34, 43, 178) (Fig. 2). The native biofilms on sprout surfaces are heterogeneous assemblages of different bacteria (as determined based on various morphotypes), yeasts, or mixtures of bacteria and yeasts (38, 43). Using conventional scanning electron microscopy, Fett and Cooke (43) imaged native biofilms that were several cell layers thick. With the use of confocal scanning laser microscopy, native biofilms on alfalfa, clover, and mung bean sprouts were estimated to have a maximum thickness of 13 μm (45). Bacteria present in biofilms of even minimal thickness most likely have greatly increased resistance toward desiccation, predation, and washing and sanitizing treatments (30,111).

Certain indigenous bacteria also may gain protection from environmental and antimicrobial stresses by internalization into plants (67, 74). Entry into plants can be passive at sites such as wounds, at areas of secondary root emergence or broken trichomes, or at natural openings such as stomata and lenticels. Active entry by the action of bacterial hydrolytic enzymes such as pectinases and cellulases can also take place. Once inside of plants, the bacteria may reside in vascular tissue, in intercellular spaces, or in intracellular locations in a latent state or may actively colonize plant tissues (74). Gagne et al. (67) found that pseudomonads and *Erwinia*-like bacteria constitute the majority (75%) of the isolates obtained from the xylems of field-grown alfalfa, similar to the pattern of predominant bacteria associated with a

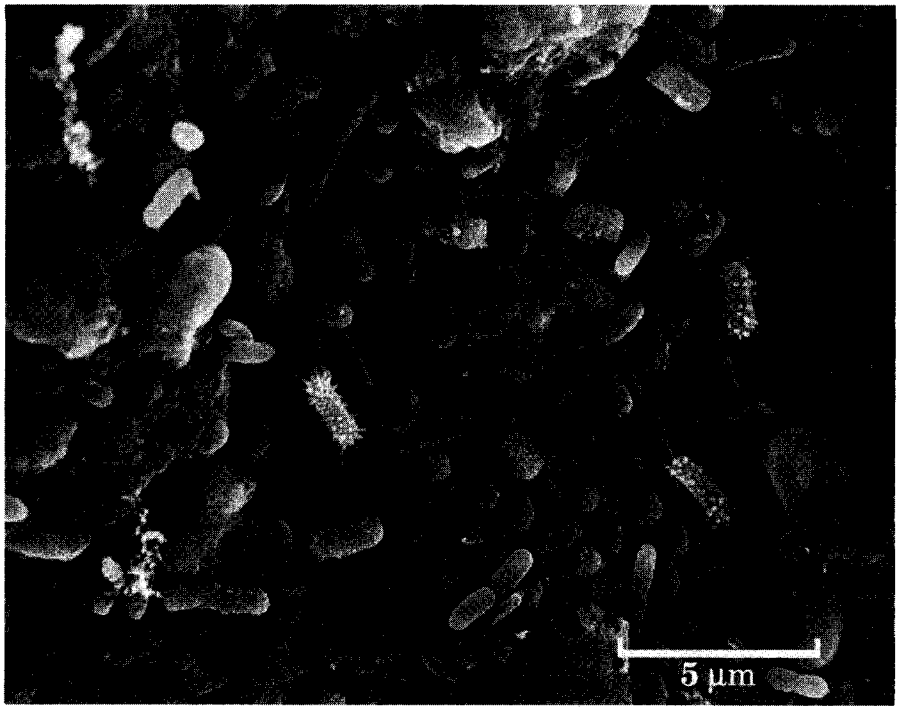

Figure 2 Scanning electron micrograph of a native biofilm on the surface of a mung bean sprout cotyledon.

variety of sprout types as noted above. Populations of bacteria isolated from the xylems ranged from 3.8 to 4.6 $\log_{10}$ CFU/g (67). Dong et al. (29) recently demonstrated that a strain of *K. pneumoniae* was a good colonizer of alfalfa roots and the interiors of the plants at 5 days into the sprouting process.

HUMAN PATHOGENS ASSOCIATED WITH SPROUTS

As noted above, consumption of contaminated raw seed sprouts has been responsible for numerous foodborne outbreaks of infection both in the United States and in other countries (for detailed listings of sprout-related outbreaks, see references 42, 103, 117, 148, 154, and 168). Sprout-related outbreaks have received much attention from regulatory bodies around the world since 1995. From 1995 through 2004, the number of annual outbreaks in the United States varied from a high of six to a low of one. The numbers of culture-confirmed cases of human illness per outbreak in the United States have ranged from 4 to almost 500. Recorded sprout-related outbreaks have also taken place in Canada, Denmark, Finland, Japan, The Netherlands,

Sweden, and the United Kingdom. Even though the great majority of sprout-related illnesses have been due to contamination with various serovars of *Salmonella,* the largest outbreak worldwide took place in Japan in 1996, with over 7,000 confirmed cases, and this outbreak was due to consumption of radish sprouts contaminated with *E. coli* O157:H7 (109, 181). The radish sprout industry in Japan has still not recovered from this devastating outbreak. Single outbreaks in the United States due to contamination of sprouts with *Bacillus cereus* and *Yersinia enterocolitica* have been recorded (131, 154). Enterotoxin-producing strains of *B. cereus* were recently isolated from 12 of 17 samples of soybean sprouts purchased in retail markets in Korea, but no associated outbreak was reported (93). In almost all outbreaks, contaminated seeds are thought to be the primary source of the bacterial pathogens based on direct isolation from the seeds and/or epidemiological evidence (117, 168). Seeds sold for sprouting are harvested from fields in numerous countries, including Australia, Burma, Canada, China, Italy, Peru, Thailand, and the United States.

Although not associated with sprout-related outbreaks, a number of other human pathogens have been isolated from sprouts. These include *Listeria monocytogenes* (163), *Aeromonas caviae* and *Aeromonas hydrophila* (19, 108), *Staphylococcus aureus* (134), *K. pneumoniae* (127, 142), and the protozoan parasites *Giardia* and *Cryptosporidium* spp. (140). In the United States, where regulatory agencies presently have a zero tolerance policy for *L. monocytogenes* on ready-to-eat foods (60), packages of sprouts have been recalled due to their potential contamination with this pathogen (53).

Several studies have demonstrated that *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes* have the potential to grow to high levels on laboratory-grown sprouts propagated from artificially inoculated seeds (3, 5, 22, 23, 82, 90, 160, 161, 167, 179). Increases of up to 100,000-fold in populations of these microbes on sprouts propagated at 20 to 30°C have been noted, with the majority of growth taking place during the first 48 h of propagation. The doubling time for *S. enterica* on sprouting alfalfa seeds is estimated to be 47 min during the initial rapid growth phase. Growth is not dependent on serovar, isolation source, or virulence characteristics (82). Maximum populations for *S. enterica* and *E. coli* O157:H7 ranging from 5 to 8 $\log_{10}$ CFU/g have been reported. Populations of *E. coli* O157:H7 on alfalfa sprouts were consistently 1 log lower than those of *S. enterica.* This finding was attributed to stronger attachment of *Salmonella* to sprout surfaces, which lessened the rinsing effect of frequent irrigation (5, 25).

For *L. monocytogenes,* maximum populations on sprouting alfalfa seeds range from 5 to 8 $\log_{10}$ CFU/g (73, 120, 146). Gorski et al. (73) documented a wide range of abilities among strains of *L. monocytogenes* to attach to alfalfa

sprout surfaces. These strain-specific differences were not related to serotype, lineage, or the original source of the pathogen (e.g., plant versus nonplant). Populations of *Salmonella, E. coli* O157:H7, and *L. monocytogenes* have been shown to be stable or to decline only slightly on contaminated sprouts stored at refrigeration temperatures (23, 82, 90, 146, 167).

The maximum populations achieved by bacterial human pathogens on sprouts do not appear to depend on the initial populations on the seeds (23, 73, 160) when the seeds are inoculated at a relatively high level. The findings indicate that there may be a maximum carrying capacity for pathogens on sprout surfaces. This may be due to the number of available attachment sites and the level of nutrients, both of which may be influenced by the nature and population of indigenous microbes. However, for seeds inoculated with lower levels, the extent of pathogen proliferation seems to be influenced by the initial inoculum dose. For example, Charkowski et al. (25) showed that, at 20°C, the level of *S. enterica* serovar Newport reached 3 $\log_{10}$ CFU/sprout on sprouts grown from seeds inoculated with 1 CFU of the pathogen/ml. On seeds inoculated with 2 $\log_{10}$ CFU/ml or higher levels, the final pathogen populations reached more than 5 $\log_{10}$ CFU/sprout.

Populations of *S. enterica* (1 to 4 $\log_{10}$ CFU/g) attained after sprouting naturally contaminated seeds by using growth conditions mimicking actual industrial propagation practices were several log units lower than those reported after using artificially inoculated seeds (1, 161). The lower final populations may be due to (i) a much lower initial pathogen level on the naturally contaminated seeds than on the artificially inoculated seeds used for such studies, (ii) a higher percentage of injured bacterial cells, and (iii) the use of different methods and increased frequency of irrigation that have a greater probability of rinsing off pathogens that were not tightly bound to the sprout surface.

Other human pathogens or opportunistic human pathogens shown to be able to multiply during growth of sprouting seed in the laboratory include *B. cereus* (attained populations of 4 $\log_{10}$ CFU/g on alfalfa and mung bean sprouts and 7.3 $\log_{10}$ CFU/g on rice sprouts [79, 128]), *K. pneumoniae* (attained populations of 5 to 6 $\log_{10}$ CFU/g on alfalfa and mung bean sprouts [124]), and *Vibrio cholerae* (attained a population of 6 $\log_{10}$ CFU/g on alfalfa sprouts [23]). Although occasionally isolated from raw sprouts, enterotoxigenic *Staphylococcus aureus* is not believed to be a food safety concern as the pathogen is unable to grow to high populations and produce enterotoxin on sprout surfaces due to competition with native microbes (117, 172).

S. enterica, E. coli O157:H7, and *L. monocytogenes* appear to preferentially colonize the roots of sprouting alfalfa seeds (25, 73). By the second day of sprouting, aggregates of *S. enterica*, but not *E. coli* O157:H7, were visualized

on alfalfa roots (24). These aggregates may or may not represent actual biofilms consisting of bacterial cells attached to one another and to the plant surface by extracellular bacterial polymeric materials.

As for several other types of produce, including lettuce and tomatoes (180), pathogens can be internalized in sprouts during plant growth presumably via uptake through the root system. Itoh et al. (89) were the first to demonstrate this phenomenon for sprouts. By using immunofluorescence and scanning immunoelectron microscopy, *E. coli* O157:H7 was shown to locate in stomata and the vascular systems of radish sprouts grown from artificially inoculated seed. This was later confirmed for *Salmonella* spp. in mung bean and alfalfa sprouts by using bioluminescent and autofluorescent transconjugant strains inoculated onto seeds or roots (28, 69, 179). As neither *Salmonella* spp. nor *E. coli* O157:H7 is known to produce plant cell wall-degrading enzymes (e.g., cellulases and pectinases), entry into sprout roots is most likely due to passive uptake at sites of injury where lateral roots emerge (29, 74).

METHODS OF DECONTAMINATION OF SPROUT SEEDS

There have been more reported studies on interventions for the elimination of bacterial human pathogens from sprout seeds and sprouts than on those from any other type of produce. Since seeds are thought to be the primary source of pathogens for sprout-related foodborne outbreaks, more potential interventions have been tested on seeds than on sprouts. Adequate sanitation of seeds presents a unique challenge in the produce area. Due to the ability of surviving bacteria to grow rapidly during the sprouting process, the goal is to completely eliminate any pathogens present on the seeds. This must be accomplished while maintaining posttreatment seed viability as well as a commercially acceptable sprout yield, appearance, and shelf life. Taking into account the nature of the commercial sprouting industry (comprising primarily small firms with 10 employees or fewer [170]) as well as the fact that some sprouts are grown by consumers at home, seed interventions should be inexpensive, present no hazard to the environment or human health, be easily applied, and preferably consist of a single step. The ideal intervention would be effective against bacterial human pathogens and parasites that have been associated with sprouts as well as viruses that have been implicated in other produce-related outbreaks (e.g., hepatitis A) but not yet associated with sprouts. For organic sprout growers, restrictions on chemical use by private organic certifying organizations and certification under the U.S. Department of Agriculture Organic Rule also need to be addressed. More expensive interventions in terms of equipment and material costs may be feasible for

application by large seed distributors before shipment of seeds to customers. In this section, we will discuss studies on antibacterial interventions for seeds destined for sprouting and point out interventions that appear to have the most potential for use by U.S. sprout growers. The reader is also referred to the recent review on this subject by Fett (42).

Chemical and Physical Interventions

A wide variety of chemical and physical interventions have been tested as stand-alone treatments for eliminating *Salmonella, E. coli* O157:H7, and *L. monocytogenes* from artificially inoculated sprout seeds. Numerous aqueous chemicals have been tested as seed-sanitizing solutions. The FDA guidance documents (49) recommend that growers treat sprouting seeds with an approved antimicrobial treatment such as 20,000 ppm of free chlorine from $Ca(OCl)_2$ immediately before sprouting. The use of chlorine at up to 20,000 ppm and that of gamma irradiation at up to 8 kGy (50) are two approved methods for sanitizing seeds before sprouting for human consumption. The use of 20,000 ppm of free chlorine is the standard against which the efficacy of other aqueous sanitizers has been compared.

Chemical interventions

In addition to chlorine [NaOCl and $Ca(OCl)_2$], natural and synthetic antimicrobials that have been tested as aqueous sanitizers for alfalfa seeds include acetic acid, acidic electrolyzed water, acidified ClO_2, acidified $NaClO_2$, $Ca(OH)_2$, calcinated calcium, Califresh-S, ClO_2, Citrex, citric acid, Citricidal, Citrobio, Environné, ethanol, Fit, H_2O_2, H_2SO_4, lactic acid, Na_2CO_3, ozonated water, thyme oil, trisodium phosphate, Tsunami, Tween 80, Vegi-Clean, and Vortexx (6, 10, 11, 12, 44, 69, 81, 92, 95, 122, 147, 149, 150, 151, 153, 159, 166, 178, 185). Supercritical CO_2 technology has also been evaluated (107). A number of aqueous chemicals have been reported to be as effective as 20,000 ppm of chlorine for reducing populations of bacterial human pathogens on artificially inoculated alfalfa seeds without significantly reducing the seed germination rate. These include 1% $Ca(OH)_2$, 1% calcinated calcium, Citrex (20,000 ppm), 8% H_2O_2, and Fit (12, 44, 81, 147, 166, 185) (Table 1). Pretreatment of seeds with surfactants or the addition of surfactants to sanitizer solutions either had no effect or improved efficacy only slightly (1 log unit or less) (40, 166, 185). The use of sanitizers at an elevated temperature (55°C) with or without sonication led to increased efficacy but also decreased the alfalfa seed germination rate (11). Presoaking of seeds in water for 1 h before treatment with 20,000 ppm of chlorine increased the killing by 2 log units but also led to a significant decrease in the seed germination rate (40). Treatment of alfalfa seeds inoculated with *E. coli*

Table 1 Comparison of selected aqueous chemical treatments with $Ca(OCl)_2$ for sanitizing alfalfa seed

Sanitizer	Concn	Treatment time (min)	Pathogen	Log_{10} reduction	Reference
H_2O_2	8%	10	*Salmonella*	3.3	81
$Ca(OH)_2$	1%	10	*Salmonella*	3.8	81
$Ca(OCl)_2$	20,000 ppm[a]	10	*Salmonella*	3.9	81
H_2O_2	8%	10	*E. coli* O157:H7	2.9	81
$Ca(OH)_2$	1%	10	*E. coli* O157:H7	3.2	81
$Ca(OCl)_2$	20,000 ppm[a]	10	*E. coli* O157:H7	2.5	81
Calcinated calcium	1%	10	*Salmonella*	2.9	185
$Ca(OCl)_2$	20,000 ppm[a]	10	*Salmonella*	2.0	185
Fit	Per manufacturer	15	*Salmonella*	2.3	12
$Ca(OCl)_2$	20,000 ppm[a]	15	*Salmonella*	2.3	12
Fit	Per manufacturer	15	*E. coli* O157:H7	>5.4	12
$Ca(OCl)_2$	20,000 ppm[a]	15	*E. coli* O157:H7	2.6	12
Citrex	20,000 ppm	10	*Salmonella*	3.6	44
$Ca(OCl)_2$	16,000 ppm[b]	10	*Salmonella*	3.4	44
Citrex	20,000 ppm	10	*E. coli* O157:H7	3.4	44
$Ca(OCl)_2$	16,000 ppm[b]	10	*E. coli* O157:H7	3.3	44

[a]Prepared in 50 mM potassium phosphate buffer, pH 7.0.
[b]Prepared in 500 mM potassium phosphate buffer, pH 6.8.

O157:H7 with a bacteriocin (colicin HU195, an E2 type) produced by a generic strain of *E. coli* at 10,000 AU/g gave a reduction in levels of one strain of greater than 5 log_{10} units, but the bacteriocin was much less effective against two other strains inoculated onto alfalfa seeds (116). The results underscore the issue of differential strain susceptibility to bacteriocins that might restrict the efficacy of bacteriocins as antimicrobials under commercial conditions.

There have been few studies on the effects of aqueous chemical sanitizers as stand-alone treatments for other sprout seed types. Fett (41) found that treatment of artificially inoculated mung bean seeds with high levels of aqueous chlorine (16,000 to 18,000 ppm) led to log reductions in levels of *E. coli* O157:H7 and *Salmonella* (4 to 5 log_{10} CFU/g) that were higher than those demonstrated for alfalfa seeds in similar previous studies in the same laboratory. Bari et al. (6) reported that treatment of mung bean seeds with acidic electrolyzed water in combination with sonication led to reductions of 4 log_{10} units. A similar treatment of radish and alfalfa seeds led to reductions of only 1.5 and 2.6 log_{10} units, respectively.

Results of combination treatments with aqueous sanitizers have rarely been reported. A sequential treatment of inoculated alfalfa seeds with aqueous

ClO_2 (25 mg/liter; 5 min), ozonated water (14.3 mg/liter; 3 min), and thyme oil suspension (5 ml/liter; 3 min) led to a 3.5- to 4-log_{10}-unit reduction in levels of *E. coli* O157:H7 and was much more effective than each of the chemicals used alone (153). However, after 3 days of sprouting of treated seeds, populations of the pathogen were almost 8 log_{10} CFU/g. Unless able to eliminate pathogens, such combination treatments may not be practical for commercial growers due to the extra costs and manipulations involved.

The comparison of efficacy data for various aqueous sanitizers is valid for studies within a laboratory using a single set of experimental protocols, but comparison of efficacy data for sanitizers tested in different laboratories may not be valid. This is due to differences in methodologies, including differences in the seed lots employed (lots with more damaged and wrinkled seeds are more difficult to sanitize [24]) (12), in the methods of seed inoculation, in the drying and storage times before treatments are applied, in the initial pathogen populations on the seeds, in the organic loads on the seeds (11), in the use of rinses before and after seed treatment, in the pHs of sanitizer solutions, in the seed-to-sanitizer volume ratios, in the methods for applying the sanitizer treatments (e.g., hand mixing versus mechanical mixing), and in media used to enumerate survivors. In addition, if injured cells are not effectively resuscitated and accounted for, the efficacy of the intervention might be overestimated.

As evidence of the degree of variability in the results obtained in different laboratories, reported reductions in levels of *Salmonella* and *E. coli* O157:H7 artificially inoculated onto alfalfa seeds treated with 16,000 to 20,000 ppm of free chlorine range from approximately 2 to 7 log_{10} CFU/g (40, 44, 69, 92, 95, 166, 185). Log reductions for the two pathogens are usually similar (1-log_{10}-unit difference or less) for the same seed treatments applied in the same laboratory. The lower-log reductions reported were most likely due, at least in part, to the low inoculum levels (2 to 3 log_{10} CFU/g) before treatment as the maximum population reductions that could be demonstrated under these conditions were 2 to 3 log_{10} units. The higher values may be due in part to inadequate rinsing or neutralization of residual chlorine after seed treatment. Some researchers have utilized chlorine solutions adjusted to a neutral pH and some have not. At a pH of 6.8 and a temperature of 20°C, approximately 75% of chlorine in solution is in the form of hypochlorous acid, with the other 25% present as hypochlorite ion, a less effective antibacterial form of chlorine (31). At more basic pHs, the percentage of the less effective hypochlorite ion increases while the percentage of hypochlorous acid decreases.

The efficacy of treatments is also dependent on the type of seed. Higher-log reductions in levels of *Salmonella* and *E. coli* O157:H7 were observed after chlorine treatment of inoculated mung bean seeds than after treatment

of alfalfa seeds (40, 41). In a recent in-depth analysis of the published literature concerning sprout seed sanitization, Montville and Schaffner (112) concluded that chemical treatment efficacy data are highly variable and the data are more variable when more published information is available. With the exception of chlorine, most chemical treatments have been examined by only a single laboratory. The one consistent finding among the different laboratories is that no single aqueous chemical treatment has proven to be capable of eliminating bacterial human pathogens from sprouting seeds without significant reductions in seed germination rates.

The use of naturally contaminated seeds for sanitization studies is preferable to that of artificially contaminated seeds, but naturally contaminated sprout seeds are usually not readily available. Thus, there have been few studies on the effectiveness of aqueous chemical sanitizers at eliminating pathogens from naturally contaminated seeds, and the results to date have been conflicting. Both Suslow et al. (164) and Fett (40) reported that treatment of alfalfa seeds naturally contaminated with *S. enterica* with 2,000 or 20,000 ppm of chlorine from $Ca(OCl)_2$ eliminated the pathogen. A similar study by Stewart et al. (161) using an identical contaminated seed lot indicated that treatment with 20,000 ppm of chlorine did not eliminate *Salmonella*. There are several possible explanations for the conflicting results, including differences in the extents of mixing during treatment, differences in the extents of contamination of the seed samples tested, the location of the pathogens on individual contaminated seeds, and the distribution of the pathogen throughout the lot.

Based on an investigation of a 1999 outbreak of salmonellosis related to contaminated clover sprouts, the use of 20,000 ppm of chlorine for seed soaking as recommended by the FDA (49) and as applied by commercial growers may not be totally effective at eliminating the risk of sprout-related outbreaks of foodborne illness (133). However, an investigation of a 1999 alfalfa sprout-related outbreak of salmonellosis indicated that sprouts produced by growers who used a chlorine seed soak were not involved in the outbreak while those produced by growers who did not use a seed decontamination step were (71). It is difficult to assess the efficacy of the chlorine treatment under commercial practice due to variability in the actual preparations and methods of application of the chlorine solutions.

Gas and vapor treatments for sanitizing seeds have also been examined, and one would expect that such treatments would have improved capabilities of reaching pathogens present deep in cracks in the seed coat and in natural openings such as the hilum. Chemicals tested include gaseous acetic acid, allyl isothiocyanate, ammonia, carvacrol, cinnamic aldehyde, eugenol, linalool, methyl jasmonate, thymol, and *trans*-anethole (28, 80, 125, 186).

Two promising treatments are gaseous acetic acid for sanitizing mung bean seeds and gaseous ammonia for sanitizing alfalfa and mung bean seeds. Treatment of mung bean seeds with gaseous acetic acid (242 μl per liter of air) for 12 h at 45°C eliminated both *Salmonella* and *E. coli* O157:H7 (initial populations of 3.7 to 6.0 $\log_{10}$ CFU/g) and resulted in a 4-$\log_{10}$-unit reduction in levels of *L. monocytogenes* from artificially inoculated seeds without a reduction in seed germination rates (28). A similar treatment of alfalfa seeds led to a significant reduction in the seed germination rate (P. Delaquis, personal communication). Treatment of inoculated alfalfa and mung bean seeds with 300 ppm of gaseous ammonia (22 h at 20°C) led to a 2- to 3-$\log_{10}$-unit reduction in levels of *Salmonella* and *E. coli* O157:H7 on alfalfa seeds and a 5- to 6-$\log_{10}$-unit reduction in levels on mung bean seeds without reducing seed germination rates (80).

Physical interventions

A variety of physical treatments have been tested as stand-alone interventions for sanitizing sprout seeds, including hot water, dry heat, gamma irradiation, hydrostatic pressure, pulsed UV light, radio frequency dielectric heating, and ultrasound (sonication). Heat treatments of planting materials, including seeds, prior to planting for the purpose of eliminating plant pathogens (sometimes referred to as thermotherapy) have been studied since at least the 1920s (106). Hot-water treatments for reducing populations of bacterial human pathogens on sprout seeds were first examined by Jaquette et al. (90). They found that a 5-min treatment in hot water at 57 to 60°C led to a 2.5-$\log_{10}$-unit decrease in the population of *S. enterica* on artificially inoculated alfalfa seeds and no appreciable reduction in the seed germination rate. However, slightly higher temperatures or longer treatment times led to significant reductions in the germination rate. Enomoto et al. (33) studied the ability of hot-water treatments to eliminate the nonpathogenic *E. coli* strain ATCC 25922 from artificially inoculated alfalfa seeds. By using a three-step process (30-min presoak at 25°C, 9-s treatment at 50°C, and 9-s treatment at 85°C), a >4-$\log_{10}$-unit reduction was achieved with no significant loss in germination rate. This reduction was greater than that obtained in the same study using 20,000 ppm of chlorine. Mung bean seeds artificially inoculated with *S. enterica* serovar Senftenberg 775W, an unusually heat-resistant strain of *Salmonella*, were also successfully treated with hot water (184). Decimal reduction times (the time of treatment to obtain a decrease of 90% in numbers of viable CFU) of 3.9, 1.9, and 0.6 min were determined for treatments at 55, 58, and 60°C, respectively. Reductions of >5 $\log_{10}$ units without reduction of the seed germination rate were obtained with the following temperature and time regimes: 55°C and 20 min; 60°C and 10 min;

70°C and 5 min; and 80°C and 2 min. Excellent results have also recently been reported by Hu et al. (83) for dry-heat treatments of mung bean seeds. Storage of seeds inoculated with cocktails of strains of *E. coli* O157:H7 or *S. enterica* serovars at 55°C for 4 or 5 days, respectively, completely eliminated initial pathogen populations of 6 $\log_{10}$ CFU/g for *E. coli* O157 and 4 $\log_{10}$ CFU/g for *Salmonella.*

On the basis of these studies, hot-water and hot-air treatments of sprout seeds appear promising; however, the commercial application of such strategies may be problematic. Even though hot-air treatments were successful at eliminating pathogens from mung bean seeds, the authors reported in the same study that the germination rate of alfalfa seeds treated in the same manner was significantly reduced. Heat treatments may be more applicable to larger sprout seed types with thicker seed coats where the storage tissues and embryo are more insulated against the damaging effects of heat. In an in-depth series of studies on the application of hot humid air (aerated steam) to seeds to control seed-borne diseases of cereals, Forsberg (61) determined that there are differences in heat tolerance levels not only among seed types but also within a seed lot due to the production and storage histories of the seeds. Seeds of low moisture content are more heat resistant. Seeds in storage for longer time periods, even under ideal conditions (low temperature and low relative humidity), undergo an aging process which reduces their ability to withstand stress, including heat. Where final seed lots ready for distribution consist of mixtures of seeds from different primary lots (as is sometimes the case in the seed sprout industry), variations in heat tolerance levels within the final lot can occur. Thus, there is often a very narrow time-temperature treatment window where pathogen levels can be significantly reduced or eliminated from a particular seed lot and the seed germination rate, quality of the plant, and yield can be maintained at acceptable levels. Because of these considerations, Forsberg (61) recommended that each seed lot be sampled at various locations and that the samples of seeds be pretested for heat tolerance before the entire seed lot is treated. If heat treatments are applied by large seed distributors, a decrease in the subsequent storage potential of the seeds may result (106).

In addition to the difficulty in maintaining the seed germination rate and the yield at acceptable levels, the ability of hot-water treatments to eliminate *S. enterica* from naturally contaminated seeds has been questioned. Suslow et al. (164) found that placing naturally contaminated seeds in hot water at up to 85°C for 1 min did not eliminate *Salmonella.* Also, a recent sprout-related outbreak of salmonellosis was reportedly due to consumption of sprouts grown from contaminated alfalfa seeds that had been treated with a proprietary hot-water treatment followed by a soak in aqueous chlorine (2,000 ppm) (188).

The FDA permits doses of ionizing radiation up to a maximum absorbed dose of 8 kGy to control human pathogens on sprout seeds (50). Treatment of sprout seeds with gamma irradiation would most likely be applied before distribution of seeds to individual sprout growers. Treatment with gamma radiation at 2 kGy can significantly reduce (2 to 3 $\log_{10}$ units) bacterial pathogen populations on sprouting seeds while maintaining adequate sprout yield and nutritional quality (37, 138, 169). Exposure to higher levels of gamma radiation are required to achieve a 5-$\log_{10}$-unit reduction on artificially inoculated seeds and to eliminate *S. enterica* from naturally contaminated seeds, but the use of higher doses is not practical due to the harmful effects on yield and sprout quality. However, a recent study by Bari et al. (6) indicated that a dry-heat treatment (50°C; 1 h) followed by exposure to gamma radiation (2 to 2.5 kGy) eliminated *E. coli* O157:H7 from artificially inoculated alfalfa, radish, and mung bean seeds (4- to 5-$\log_{10}$-unit reductions) without decreasing seed germination rates. The combination treatment did significantly decrease the lengths of both radish and mung bean sprouts, but not that of alfalfa sprouts, after 4 days of growth. A problem with the commercial application of gamma radiation for sanitizing seeds is the phenomenon of uneven absorbance of dosages by seeds at different locations in the treatment chamber. The dose absorbed by seeds located near the exterior of the chamber is higher than the dose absorbed by seeds at the center of the chamber. This unevenness of exposure might lead to variable effects on pathogen reduction within the treated batch of seeds as well as differential effects on the subsequent seed germination rate, sprout quality, and yield.

High-hydrostatic-pressure treatments may be useful for sanitizing a limited number of types of sprout seeds before distribution. Exposure to high pressure (250 to 300 MPa for 15 min at 20°C) significantly reduced (>6 $\log_{10}$ units) populations of *S. enterica*, *E. coli*, and *Listeria innocua* on garden cress seeds, but the treatment was highly detrimental to the germination of radish, mustard, and sesame seeds (191). High-hydrostatic-pressure treatment of alfalfa seeds (575 MPa for 2 min or 475 MPa for 2 to 8 min at 40°C) was less effective at reducing *E. coli* O157:H7 and *L. monocytogenes* populations and led to a significant reduction in the germination rate (4).

Combined chemical and physical treatments

There have been few published studies on combining chemical and physical treatments for seed sanitization. Combining two or more antimicrobial treatments that have different modes of action may result in increases in killing that are either additive or synergistic. Synergistic effects occur if the first treatment leads to a large number of injured cells that have increased susceptibility to the second antimicrobial intervention. Bari et al. (6) reported

that a combination treatment with dry heat (50°C; 1 h), hot acidic electrolyzed water, and sonication eliminated (a 4.6-$\log_{10}$-unit reduction) *E. coli* O157:H7 from inoculated mung bean seeds without a reduction in germination rate or average sprout length after 4 days of growth but was unable to eliminate the pathogen from inoculated radish and alfalfa seeds. The dry-heat treatment alone and a combined treatment with dry heat followed by hot acidic electrolyzed water were not as effective. Sharma et al. (150) reported that treatment of ozone-sparged alfalfa seeds with dry heat (60°C; 3 h) was more effective than ozone treatment alone and resulted in a 4- to 4.8-$\log_{10}$-unit reduction in the *E. coli* O157:H7 population without compromising the germination rate. Combining sonication with hot aqueous chemical treatments led to slight increases in effectiveness (147). The combined effects of ionizing radiation treatments applied before seed distribution followed by various chemical treatments that could be applied by growers immediately before sprouting of seeds should be evaluated.

Biological Interventions

There have been few studies on the use of biological control (commonly referred to as competitive exclusion or probiotics in the food microbiology literature) strategies for controlling the growth and survival of human pathogens on produce, including sprouting seeds. In contrast, biological control has been intensively studied as a means of controlling plant diseases since at least the early 1920s (20) and, more recently, has been investigated for controlling human pathogens in chicks, swine, calves, meat, and dairy products (13, 118, 173). For control of human pathogens in food animals or in foods, single microbial strains or defined or undefined consortia of microbes have been tested as antagonists. Several commercial products are on the market for use in controlling the colonization of newly hatched chicks with *Salmonella*. One such product is named PREEMPT and is a mixture of 29 different bacterial isolates originally obtained from the cecal contents and cecal tissues of adult chickens (118). Effective biological control products for controlling growth of bacterial human pathogens on sprouting seeds and sprouts postharvest would be very useful for organic as well as conventional growers.

As demonstrated by several studies mentioned previously, bacterial human pathogens are surprisingly good competitors on sprout surfaces and can grow to high levels on growing sprouts after germination of artificially inoculated or naturally contaminated seeds. However, competitive exclusion does occur naturally on sprouts as evidenced by little growth (1 $\log_{10}$ unit or less) of bacterial human pathogens inoculated onto sprouts 1 to 5 days into the sprouting process (23, 146). Thus, the key for effective control is ensuring the presence

of high levels of effective competitive microflora on the seed at the time of sprouting.

Several lactic acid bacteria isolated from alfalfa seeds and sprouts were demonstrated to be highly inhibitory toward *S. enterica, E. coli* O157:H7, and *L. monocytogenes* in vitro (121, 187); however, when the inhibitory isolate *Lactobacillus lactis* SP 26 was applied to seeds artificially inoculated with *L. monocytogenes* and the seeds sprouted, outgrowth of the pathogen was reduced by only 1 $\log_{10}$ unit (120). As mentioned above, lactic acid bacteria usually do not constitute a significant proportion of the native bacteria present on sprouting seeds and may not be able to outcompete or exhibit significant antibiosis against bacterial human pathogens on the growing plant surface. There is, however, a commercial product based on lactic acid bacteria that is sold in Japan for controlling bacterial human pathogens on sprouting seeds.

The proof that competitive exclusion is a viable intervention against *Salmonella* outgrowth on sprouts comes from the work of Matos and Garland (105). Addition of *Pseudomonas fluorescens* 2-79 to the seed soaking solution at 8 $\log_{10}$ CFU/ml led to a 4-$\log_{10}$-unit reduction in the population of *Salmonella* at days 1 and 3 of sprouting of artificially inoculated alfalfa seeds. Whole microbial communities isolated from alfalfa sprouts purchased at a supermarket and added to the seed soaking solution were not as effective at inhibiting outgrowth of *Salmonella* at days 1 and 3 but gave greater log reductions at day 7 of sprouting. There was no adverse effect of inoculation with strain 2-79 on sprout appearance. Strain 2-79 was originally isolated from the rhizosphere of a field-grown wheat plant and is a known biological control agent against the wheat root pathogen *Gaeumannomyces graminis* var. *tritici* (171). Recent studies by Fett (W. F. Fett, unpublished results) using a collection of mutants of strain 2-79 confirmed antibiosis of strain 2-79 against *S. enterica* both in vitro and in situ on sprouting alfalfa seeds and also indicated that the production of iron-binding siderophores and the antibiotic phenazine-1-carboxylic acid (the two primary antifungal metabolites produced by this strain) is not responsible for inhibitory activity. Such biocontrol strategies may also be effective for controlling bacterium-mediated spoilage of germinating seeds (32) and, with the use of endophytic antagonists, may be effective against internalized bacterial human pathogens (26).

Another biological approach to controlling pathogen survival and growth on sprouting seeds is the application of bacteriophage. The use of lytic phage for the control of bacterial plant pathogens is an active area of research (72). The application of lytic phage is also being studied for the control of bacterial human pathogens on fresh-cut produce. Spraying a lytic phage (8 $\log_{10}$ CFU/ml) onto inoculated fresh-cut honeydew melon was demonstrated to reduce populations of *L. monocytogenes* to below detectable levels, and no sig-

nificant outgrowth of any survivors occurred during subsequent storage for 7 days at 10°C (97). Pao et al. (123) tested the abilities of two lytic phages to inhibit outgrowth of *S. enterica* on sprouting broccoli and radish seeds. Application of phage at approximately 6 to 7 $\log_{10}$ PFU/ml at the time of germination was not highly effective (less than a 1.5-$\log_{10}$-unit reduction after 24 h) against susceptible *S. enterica* serovars, and there was no activity against resistant *S. enterica* serovars. The host specificity of lytic phage would necessitate the use of phage mixtures under commercial practice.

A general observation concerning most antibacterial intervention studies published to date is that the effects of antibacterial treatments on seed germination rates only are reported. From a grower's perspective, the appearance, yield, and shelf life are also important parameters to consider.

INTERVENTIONS DURING SPROUTING OR POSTHARVEST

During sprouting, growing plants are frequently irrigated to prevent desiccation and to cool sprouts grown in large bins (primarily mung bean). The addition of antimicrobials to the irrigation water is a possible means of reducing the growth of bacterial human pathogens; however, there have been few published studies in this area compared to the number of studies on potential interventions for seeds. Spray irrigation of sprouting rice with chlorinated water (100 ppm) every 6 h was not effective at reducing populations of *B. cereus* or *L. innocua* (129). Daily spray irrigation with chlorine (100 ppm) reduced populations of *S. enterica* and *V. cholerae* by 2 $\log_{10}$ units or less on sprouting alfalfa seeds (22, 69). Taormina and Beuchat (167) tested the addition of several antibacterial chemicals to the spray irrigation water, including NaOCl (up to 2,000 ppm), $Ca(OCl)_2$ (up to 2,000 ppm), and acidified $NaClO_2$ (up to 1,200 ppm). None of the chemicals tested significantly reduced populations of *E. coli* O157:H7 on sprouting alfalfa seeds. When sprouting alfalfa seeds were rinsed with aqueous ClO_2 (25 ppm) or ozonated water (9 ppm) after 48 or 72 h of sprouting, populations of *E. coli* O157:H7 were not reduced (153). Rinsing with thyme oil (5 ppm) alone or in sequence with ClO_2 and ozonated water at 24 to 48 h into the sprouting process led to reductions in pathogen populations on the sprouts of up to 2 $\log_{10}$ units, but the same treatments were ineffective when applied to sprouts at 72 h (153). This may indicate that the pathogen had either formed biofilms or become part of biofilms formed by native microflora (38, 43, 45) and/or was internalized and thus resistant or not exposed to the sanitizing rinses. The effects of such a sequential treatment on sensory qualities of treated alfalfa sprouts were not evaluated.

The addition of antimicrobials to the irrigation water would not be compatible with the FDA recommendation to test spent irrigation water for

Salmonella and *E. coli* O157:H7 (49). Pathogens rinsed from the sprout surface and thus suspended in an aqueous solution would be more easily killed than would pathogenic cells still tightly attached to the sprout surfaces. Thus, a negative test result for the spent irrigation water would not accurately reflect the microbial safety status of the sprouts.

The reduction or elimination of bacterial human pathogens present on sprouts postharvest has also proven problematic. Few antimicrobial interventions have been reported to be capable of achieving a 5-$\log_{10}$-unit reduction or greater without an adverse impact on quality. Rinsing with water reduces bacterial human pathogens and native microbes on sprouts by 1 $\log_{10}$ unit or less (5, 126). Washing of alfalfa sprouts with acidic electrolyzed water (50 ppm of available chlorine) for 64 min resulted in a 3-$\log_{10}$-unit reduction in the *E. coli* O157:H7 population with no reported change in sprout appearance (149). Treatment with acidic electrolyzed water (84 ppm of available chlorine; 10 min) in combination with sonication and removal of seed coats led to a 3.3-$\log_{10}$-CFU/g reduction in *Salmonella* populations compared to the control (92). For *S. enterica* and *L. monocytogenes* on mung bean sprouts, a 10-min treatment with chlorous acid (268 ppm) resulted in an approximate 5-$\log_{10}$-unit reduction without adversely affecting visual quality (96).

For pathogens that are internalized, treatment with gamma radiation may be the only effective intervention. Rajkowski and Thayer (136) demonstrated that gamma irradiation to a minimum dose of 0.5 kGy could eliminate *S. enterica* from alfalfa sprouts grown from naturally contaminated seeds. Subsequently, exposure of uninoculated alfalfa sprouts to 2 kGy of gamma radiation was reported to extend the shelf life by more than 10 days without a reduction in nutritional quality (36, 137). Schoeller et al. (146) found that irradiation of alfalfa sprouts inoculated with *L. monocytogenes* (6 $\log_{10}$ CFU/g) with 3.3 kGy of beta radiation (electron beam) eliminated the pathogen without an adverse effect on quality. *Salmonella* and *E. coli* O157:H7 were eliminated from inoculated mung bean and radish sprouts with doses of 1.5 and 2.0 kGy, respectively, and acceptable quality was maintained (7). However, the use of gamma or beta radiation for eliminating bacterial human pathogens from sprouts has not yet received regulatory approval in the United States.

Few synthetic chemicals are allowable for use on organic produce under the USDA Agricultural Marketing Service's National Organic Program Regulations (176). These include ethanol and chlorine materials such as calcium and sodium hypochlorite and chlorine dioxide, hydrogen peroxide, and peracetic acid (for disinfecting equipment, seeds, and asexually propagated materials). Residual chlorine levels in the wastewater should not exceed the

maximum residual disinfectant limit under the Safe Drinking Water Act, presently 4 ppm (34). Treatment with ionizing radiation is not allowed.

PATHOGEN SAMPLING AND DETECTION

Testing of Sprouts and Spent Irrigation Water for the Presence of Pathogens

Since the recommended use of 20,000 ppm of $Ca(OCl)_2$ for sanitizing seeds does not guarantee the elimination of pathogens (95, 161, 166), a microbiological testing program is also recommended to ensure sprout safety (49, 117). The presence of fecal coliforms is not a good indicator of recent fecal contamination because the coliforms are often found on plant surfaces as part of the native microflora (127, 193); therefore, the pathogens need to be targeted. In 1999, the FDA issued guidance to the sprout industry, recommending the testing of each production batch for the presence of *Salmonella* and *E. coli* O157:H7, the two most common agents of sprout-associated illness (49).

The optimal time for testing sprouts is when the pathogen level is highest but also when it is early enough to ensure that results are obtained before the product is shipped. The sprouting process encourages microbial proliferation and provides a natural environment for amplifying pathogens. Typically, the greatest increase in pathogen populations occurs during the first 24 h of sprouting, and maximal levels are achieved at 48 h (23, 25, 65, 68, 82, 90, 160, 161, 167). Table 2 summarizes published data regarding pathogen levels during sprouting at 24 h versus 48 h under various experimental growing conditions. In most cases, the levels of pathogens observed at 24 h closely approached those observed at 48 h. However, the differences between the levels of *Salmonella* at 24 and 48 h seemed to be greater (up to 2.3 $\log_{10}$ units) when naturally contaminated seeds were used than when artificially inoculated seeds were used. The FDA recommends sampling for microbiological testing 48 h after starting production (49).

Sampling of sprouts during seed germination in commercial settings presents certain difficulties. The distribution of pathogens in food is often nonhomogeneous; therefore, it is necessary to obtain multiple samples at various sites to ensure good representation of the entire production batch. Taking samples of sprouts may be difficult because the equipment used (e.g., rotary drums) is not designed for easy access and, in some cases, removal of sprouts from the production batch at various locations may disrupt proper seed germination (J. Louie, personal communication). Furthermore, additional equipment is required for preparing the samples for analysis (e.g., a homogenizer or stomacher). On the other hand, during production, the sprouts are

Table 2 Studies on the growth of bacterial human pathogens on sprouting seeds

Sprouting apparatus	Seed status (lot)	Pathogen	Log CFU/g of seeds (day 0)	Log CFU/g of sprouts		Reference
				Day 1 (24 h)	Day 2 (48 h)	
Mini drum, (25°C)	Naturally contaminated (COA98)	*S. enterica* serovar Muenchen	−1.5	2.0	2.0	65
Mini drum (30°C)	Naturally contaminated (45197)	*S. enterica* serovar Mbandaka	−2.4 (run 1), −1.5 (run 2)	−0.6 (run 1), 0.5 (run 2)	−1 (run 1), 1.8 (run 2)	65
Glass jars	Naturally contaminated (COA98)	*S. enterica* serovar Muenchen	−1	1.4	2.3	65
Glass jars	Naturally contaminated (45197)	*S. enterica* serovar Mbandaka	−1.6	2.6	3.1	65
Glass jars	Naturally contaminated (A)	*Salmonella*	−1.2	1	2.5	161
Glass jars	Naturally contaminated (B)	*Salmonella*	Below detection limit	1.7 to 2.2	2 to 4.5	161
Plastic container	Artificially inoculated	*S. enterica* serovar Stanley	3.2	6	7	10
Stainless-steel tray	Artificially inoculated	*S. enterica* serovar Typhimurium	3.4	5	5.4	23
Test tube	Artificially inoculated	*S. enterica* serovar Newport	1.8	4.7	6.3	25
Easy Green sprouting unit	Artificially inoculated	*S. enterica* serovar Cubana	0	5.7	5.7	82
Screen tray	Artificially inoculated; treated with 20,000 ppm of chlorine	*S. enterica* serovar Stanley	3.6	6.9	7.9	68

Plastic box with drain holes	Artificially inoculated; sprayed with water	*E. coli* O157:H7	3.1	5.0	4.8	167
Stainless-steel tray	Artificially inoculated	*E. coli* O157:H7	3.1	5.7	5.7	23
Glass jars	Artificially inoculated	*E. coli* O157:H7	3.9 (high inoculum); 1.9 (low inoculum)	6 (high inoculum); 4.5 (low inoculum)	5.5 (high inoculum); 5.3 (low inoculum)	160
Test tube	Artificially inoculated	*E. coli* O157:H7	2.7	3.8	4.3	25
Mini drum with recycled irrigation water	Artificially inoculated	*L. monocytogenes*	2.5	6	6	120
Glass jars	Artificially inoculated	*L. monocytogenes*	2.5	5.5	7	146

constantly irrigated with water, which gathers microorganisms as it passes through the sprouting seeds. This spent irrigation water provides a better representation of the microbial population in the production batch. The sampling and microbial testing of spent irrigation water are relatively simple compared to those of sprouts. In addition, the sizes of the water samples can be easily scaled up to increase the chance of detecting the pathogens. Populations of *Salmonella* and *E. coli* O157:H7 in spent irrigation water are highly correlated with the populations on the germinating seeds (naturally contaminated or artificially inoculated), with populations approximately 1 $\log_{10}$ lower in the spent irrigation water (64, 82, 160). When alfalfa seeds naturally contaminated with *Salmonella* were sprouted, the numbers of confirmed positive samples were the same for the sampled sprouts and the spent irrigation water (161), suggesting that water analysis may accurately indicate the presence of pathogens on the sprouts.

Detailed protocols for the sampling and microbial testing of spent irrigation water and sprouts for the pathogens *Salmonella* and *E. coli* O157:H7 were included in the 1999 FDA guidance documents for the commercial production of sprouts (49, 157). The FDA recommended that, whenever possible, spent irrigation water rather than sprouts be sampled and that each production batch be sampled independently. Briefly, 1-liter samples of spent irrigation water should be aseptically collected as early as 48 h into the sprouting process and then subjected to microbiological testing with inclusion of an enrichment step. For instances in which collection of spent irrigation water is not feasible (e.g., for sprouts grown in soil), the sprouts should be tested. They should be aseptically sampled at different locations in the drum or growing trays and homogenized in a blender or stomacher prior to analysis. Testing by an independent, certified laboratory is preferred.

The perishable nature of the sprouts requires a quick turnaround of test results, and the use of rapid pathogen test kits was recommended by the FDA (49). Although many rapid test kits were commercially available at the time the FDA guidance was issued, few had undergone the extensive collaborative testing required for official approval. For *Salmonella*, the Assurance Gold enzyme immunoassay (Biocontrol, Inc., Bellevue, Wash.; AOAC official method 999.08) and the Visual Immunoprecipitate VIP immunoassay (Biocontrol, Inc.; AOAC official method 999.09) were available. For *E. coli* O157, only the Visual Immunoprecipitate VIP enterohemorrhagic *E. coli* assay (Biocontrol, Inc.) was officially approved (AOAC official method 996.09), but additional experience specifically with sprout testing by researchers using the Reveal for *E. coli* O157:H7 (Neogen Corporation, Lansing, Mich.) provided support for including it as an alternative test in the FDA guidance document (49). Despite the high microbial levels in sprouts

and spent irrigation water, pathogen enrichment is necessary even for the rapid test kit procedures, and there have been no direct (i.e., nonenrichment) methods validated. The FDA guidance (49) detailed the enrichment steps for *E. coli* O157:H7 (enrichment in modified buffered peptone water supplemented with acriflavin, cefsulodin, and vancomycin) and for *Salmonella* (preenrichment in buffered peptone water supplemented with novobiocin, followed by enrichments in Rappaport-Vassiliadis and tetrathionate broths).

The efficacy of these rapid test kits for detection of *E. coli* O157:H7 and *Salmonella* in sprout irrigation water has been evaluated, and some kits have been compared with official conventional culture methods as described in the FDA's *Bacteriological Analytical Manual* (BAM) (47). The Reveal for *E. coli* O157:H7 and the VIP enterohemorrhagic *E. coli* tests could detect the presence of *E. coli* O157:H7 in 100 and 72%, respectively, of 36 samples inoculated at levels of 0.6 to 3.6 CFU/ml (182). The Assurance Gold for *Salmonella* enzyme immunoassay and the VIP for *Salmonella* test successfully detected 100% of all 66 samples inoculated with 0.67 to 3.6 CFU of the pathogen/ml, whereas the BAM method detected *Salmonella* in only 49 of the 66 inoculated samples (174).

In addition to those recommended by the FDA, many other methods have been evaluated for pathogen testing of sprouts. Most of the methods evaluated have targeted *Salmonella* and *E. coli* O157 (the two most common pathogens involved in illness outbreaks from sprouts) and may be categorized as conventional culture methods, immunoassay-based methods, and nucleic acid-based methods. The culture methods include the FDA BAM protocol (174), the use of various enrichment and recovery media (119, 182, 189), and membrane filter plating on selective agar media (160). The immunoassay-based methods include the use of several commercial lateral flow devices (e.g., VIP, Reveal, and Quix), an antibody-direct epifluorescent filter (Ab-DEFT) technique (160), an immunomagnetic capture and time-resolved fluorescence method (175), an antibody-based fiber-optic evanescent-wave biosensor system (94), the use of a multiarray-based immunosensor (165), and an electrochemical sandwich immunoassay (114). Nucleic acid-based tests evaluated include the GENE-TRAK *Salmonella* direct labeled probe (DLP) assay (162), the TaqMan *E. coli* O157:H7 PCR assay (62), multiplex PCR (63, 98), the BAX PCR assay (152, 162, 163), and real-time PCR (101). Table 3 summarizes the performance of most published methods with respect to the enrichment conditions used, the recovery protocol used, and the limit of detection.

The fiber-optic-based biosensor system relies on a polyclonal antibody for capturing *Salmonella* in spent irrigation water and then a monoclonal antibody for detection (94). When assays were done at 67 h into the sprouting

Table 3 Methods for detection of pathogens in sprouts, spent irrigation water, and seeds

Method(s)	Pathogen	Sample tested	Enrichment conditions[a]	Recovery improvement step(s)[b]	Detection limit	Reference
Culture based						
BAM protocols	*Salmonella* spp.	Spent irrigation water (alfalfa)	Preenrichment in lactose broth, 35°C, 22–26 h; selective enrichment in RV&TT, 42°C, 22–26 h	Plating on XLD, BS, HE	Positive for 39 of 66 samples inoculated at levels of 0.6–3.6 CFU/ml	174
	Salmonella spp.	Spent irrigation water and sprouts (alfalfa)	Preenrichment in lactose broth, 35°C, 22–26 h; selective enrichment in RV&TT, 42°C, 22–26 h	Plating on XLD, BS, HE	Positive for 100% of 64 samples inoculated at levels of 1–180 CFU/g	162
	E. coli O157:H7	Radish sprouts	Enrichment in mEC + n, 42°C, 18 h	IMS followed by plating on TC-SMAC agar, BCM 0157 agar, and CHROMagar O157	Positive for more than 90% of 80 samples inoculated at a level of 20.4 CFU/25 g	119
	E. coli O157:H7	Spent irrigation water (alfalfa)	Enrichment in mBPW + ACV at 42°C and 140 rpm for 24 h and EEB (1/4 concn of cefixime) at 37°C and 140 rpm for 24 h	Plating on TC-SMAC	Positive for 50 to 58% of 36 samples inoculated at levels of 0.41–1.34 CFU/ml	182
	E. coli O157:H7	Spent irrigation water (alfalfa)	Enrichment in mBPW + ACV at 42°C and 140	IMS and plating on TC-SMAC	Positive for 100% of 36 samples inoculated at	182

			rpm for 24 h		levels of 0.41–1.34 CFU/ml	
	E. coli O157:H7	Alfalfa sprouts	Enrichment in EEB, EEB (1/4 concn of cefixime), mEC at 37°C, 24 h with agitation; mBPW or mBPW + ACV, 42°C, 24 h with agitation	IMS and plating on TC-SMAC	Positive for 13 to 14 of 15 samples inoculated at levels of 0.12–0.42 CFU/g	183
	E. coli O157:H7	Alfalfa seeds	Enrichment in mTSB	Soaking of seeds for 1 h; IMS postenrichment		189
Membrane filter plating on BCM	*E. coli* O157:H7	Spent irrigation water (alfalfa)	ND	Plating on selective medium (BCM O157)	Not determined	160
	E. coli O26	Radish sprouts	mEC + n, 42°C, 18 h	IMS; plating on Rainbow agar O157 plus novobiocin	Positive for 9 of 9 samples inoculated at a level of 5.9 CFU/25 g	77
	E. coli O26	Alfalfa sprouts	Enrichment in mEC + n, 42°C, 18 h	IMS and plating on cefixime-tellurite O26 medium	Positive for 5 of 10 samples inoculated at a level of 2 CFU/25 g	78

(continued)

Table 3 Methods for detection of pathogens in sprouts, spent irrigation water, and seeds *(continued)*

Method(s)	Pathogen	Sample tested	Enrichment conditions[a]	Recovery improvement step(s)[b]	Detection limit	Reference
Immunoassay based						
Lateral flow device VIP	*Salmonella* spp.	Spent irrigation water (alfalfa)	Preenrichment in BPW + n, 35°C, 18–26 h; selective enrichment in RV&TT, 42°C, 5–8 h; post-enrichment in TSB + n, 42°C, 16–20 h	ND	Positive for 100% of 66 samples inoculated at levels of 0.6–3.6 CFU/ml	174
	E. coli O157:H7	Spent irrigation water and sprouts (alfalfa)	ND	ND	6–7 $\log_{10}$ CFU/g within 20 min	160
	E. coli O157:H7	Spent irrigation water (alfalfa)	Enrichment in mBPW + ACV at 42°C, 140 rpm, 24 h; EEB (1/4 concn of cefixime) at 37°C, 140 rpm, 24 h	ND	Positive for 72 to 75% of 36 samples inoculated at levels of 0.41–1.34 CFU/ml	182
	E. coli O157:H7	Alfalfa sprouts	Enrichment in mBPW + ACV, 42°C, 24 h with agitation (most effective enrichment)	ND	Positive for 9 of 15 samples inoculated at levels of 0.12–0.42 CFU/g	183
Lateral flow device Reveal	*E. coli* O157:H7	Spent irrigation water and sprouts (alfalfa)	ND	ND	5–6 $\log_{10}$ CFU/g within 20 min	160

	E. coli O157:H7	Spent irrigation water (alfalfa)	Enrichment in mBPW + ACV, 42°C, 140 rpm, 24 h; EEB (1/4 concn of cefixime), 37°C, 140 rpm, 24 h	ND	Positive for 100% of 36 samples inoculated at levels of 0.41–1.34 CFU/ml	182
Lateral flow device Quix	*E. coli* O157:H7	Spent irrigation water (alfalfa)	No enrichment or enrichment in BHI at room temp or 37°C	ND	Cannot be determined	62
Enzyme-linked immunosorbent assay Assurance Gold	*Salmonella* spp.	Spent irrigation water (alfalfa)	Preenrichment in BPW + n, 35°C, 18–26 h; selective enrichment in RV&TT, 42°C, 5–8 h; postenrichment in TSB + n, 42°C, 16–20 h	ND	Positive for 100% of 66 samples inoculated at levels of 0.6–3.6 CFU/ml	174
	Salmonella spp.	Spent irrigation water and sprouts (alfalfa)	Preenrichment in BPW + n, 35°C, 18–26 h; selective enrichment in RV&TT, 42°C, 5–8 h; postenrichment in TSB + n, 42°C 16–20 h	ND	Positive for 100% of 64 samples inoculated at levels of 1–180 CFU/g	174
Time-resolved fluorescence-based immunoassay	*Salmonella* spp.	Spent irrigation water and sprouts (alfalfa)	Enrichment in BHI at 37°C, 4 h, 160 rpm	IMS	Cannot be determined	175

(continued)

Table 3 Methods for detection of pathogens in sprouts, spent irrigation water, and seeds *(continued)*

Method(s)	Pathogen	Sample tested	Enrichment conditions[a]	Recovery improvement step(s)[b]	Detection limit	Reference
	E. coli O157:H7	Spent irrigation water and sprouts (alfalfa)	Enrichment in BHI, 37°C, 4 h, 160 rpm	IMS	Cannot be determined	175
Ab-DEFT	*E. coli* O157:H7	Spent irrigation water and sprouts (alfalfa)	ND	Membrane filtration	3.5 $\log_{10}$ CFU/g within 30 min	160
Evanescent wave-based multianalyte array biosensor	*S. enterica* serovar Typhimurium	Sprout homogenate and spent irrigation water (alfalfa)	ND	ND	6.6 $\log_{10}$ CFU/g (sprout) or 5.6 $\log_{10}$ CFU/ml (rinse water) within 15 min	165
Fiber-optic biosensor (RAPTOR)	*S. enterica* serovar Typhimurium	Spent irrigation water (alfalfa)	ND	ND	Cannot be determined	94
Fiber-optic biosensor (Analyte 2000)	*E. coli* O157:H7	Spent irrigation water (alfalfa)	ND	ND	Consistent positives only for samples inoculated at a level of 6.0 $\log_{10}$ CFU/ml	102
Disposable electrochemical biosensor	*E. coli* O157:H7	Alfalfa sprout homogenate	ND	ND	81 CFU/ml (within 6 min)	114
Nucleic acid based						
PCR-based TaqMan *E. coli* O157:H7 assay	*E. coli* O157:H7	Spent irrigation water (alfalfa)	No enrichment and enrichment in BHI, 37°C, 18 h	ND	Cannot be determined	62

BAX PCR	*Salmonella* spp.	Spent irrigation water and sprouts (alfalfa)	Preenrichment in lactose broth, 35°C, 22–26 h; postenrichment in BHI, 35°C, 3 h	ND	Positive for 90.6% of 64 samples inoculated at levels of 1–180 CFU/g	162
	S. enterica serovar Enteritidis	Alfalfa sprouts	Preenrichment in BPW, 37°C, overnight; postenrichment in BHI, 3 h, 37°C	ND	Positive for 5 of 6 samples inoculated at 1 CFU/25 g	152
	S. enterica serovar Enteritidis	Alfalfa sprouts	Preenrichment in mTSB, 37°C, 24 h, 150 rpm; postenrichment in BHI, 37°C, 3 h	ND	Positive for 12 of 12 samples inoculated at a level of 10 CFU/25 g	163
	S. enterica serovar Typhimurium	Alfalfa sprouts	Preenrichment in lactose, 37°C, 24 h; postenrichment in BHI, 37°C, 3 h	ND	Positive for 4 of 9 samples inoculated at a level of 4 CFU/25 g	102
	Salmonella spp.	Alfalfa seeds	Enrichment in BPW, 8 h, 37°C	IMS	Positive for 4 or 5 samples inoculated with 2 CFU of untreated *Salmonella*/25g; positive for 3 or 5 samples inoculated with 2–3 CFU of heat-injured *Salmonella*/25 g	100

(continued)

Table 3 Methods for detection of pathogens in sprouts, spent irrigation water, and seeds *(continued)*

Method(s)	Pathogen	Sample tested	Enrichment conditions[a]	Recovery improvement step(s)[b]	Detection limit	Reference
	E. coli O157:H7	Alfalfa sprouts	Enrichment in EEB, 24 h with shaking, 37°C	ND	Positive for 1 of 6 samples inoculated at a level of 10 CFU/25 g	152
	E. coli O157:H7	Alfalfa sprouts	Preenrichment in mTSB, 37°C, 24 h, 150 rpm; postenrichment in mEC, 42°C, overnight at 180 rpm	ND	Positive for 23 of 23 samples inoculated at a level of 10 CFU/25 g	163
	L. monocytogenes	Alfalfa sprouts	Preenrichment in Demi-Fraser broth, 20°C, 22–24 h; selective enrichment in MOPS-BLEB, 37°C, 20–24 h	ND	Positive for 4 of 6 samples inoculated at 1 CFU/25 g	152
	L. monocytogenes	Alfalfa sprouts	Preenrichment in Demi-Fraser broth, 30°C, 22–24 h; selective enrichment in MOPS-BLEB, 37°C, 20–24 h	ND	Positive for 12 of 12 samples inoculated at a level of 10 CFU/25 g	163
Molecular beacon real-time PCR	*S. enterica* serovar Typhimurium	Alfalfa sprouts	Enrichment in BPW, 37°C, 18 ± 2 h	ND	Positive for 5 of 9 samples inoculated at a level of 4 CFU/25 g	102

Multiplex PCR	*E. coli* O157:H7		Enrichment in mEC + n, 37°C, 24 h, 150 rpm	IMS; use of PrepMan for DNA extraction	1 CFU/g	63
	E. coli O157:H7 and *Salmonella* and *Shigella* spp.	Alfalfa sprouts	Enrichment in BHI, 37°C, 24 h	ND	1.9 $\log_{10}$ CFU/g	99

[a]RV&TT, Rappaport-Vassiliadis and tetrathionate broths; mEC + n, modified EC broth plus novobiocin; mBPW, modified buffered peptone water; ACV, acriflavin-cefsulodin-vancomycin; EEB, enterohemorrhagic *E. coli* enrichment broth; mEC, modified EC broth; mTSB, modified trypticase soy broth; ND, not done; BPW + n, buffered peptone water plus novobiocin; TSB + n, trypticase soy broth plus novobiocin; BHI, brain heart infusion; BPW, buffered peptone water; MOPS-BLEB, 3-[*N*-morpholino]propanesulfonic acid-buffered *Listeria* enrichment broth.

[b]XLD, xylose-lysine-desoxycholate agar; BS, bismuth sulfite agar; HE, Hektoen enteric agar; IMS, immunomagnetic separation.

process, *Salmonella* could be detected in 20 min without an enrichment step in the spent irrigation water after germination of alfalfa seeds inoculated with as few as 50 CFU of the pathogen/g (94). Unfortunately, the researchers failed to indicate the level of pathogens in the tested water, and thus it is difficult to estimate the detection limit of the biosensor. A similar optic-based biosensor, the Analyte 2000, was evaluated for detection of *E. coli* O157:H7 in sprout irrigation water (102). Although the sensor was able to detect *E. coli* O157:H7 in inoculated sprout irrigation water at levels as low as 1 to 10 CFU/ml, signal strength varied greatly among probes tested with the same samples. Consistent, positive reactions occurred only for samples containing *E. coli* O157:H7 at levels above 6 $\log_{10}$ CFU/ml. False positives were frequently observed. Taitt et al. (165) evaluated an evanescent wave-based, multianalyte array biosensor for detection of *Salmonella* in a variety of food matrices. This biosensor employed a 15-min sandwich immunoassay protocol and gave a detection limit of 5.6 $\log_{10}$ CFU/ml in spiked sprout rinse.

Difficulties in determining the sensitivities of published methods also exist in other cases. For example, it was reported that the time-resolved fluorescence-based immunoassay (175) could detect the presence of *E. coli* O157:H7 in seeds inoculated at a level of 4 CFU/g. However, the actual detection procedure was performed on the spent irrigation water collected after allowing the inoculated seeds to sprout for 48 h. Because the sprouting process may encourage pathogen proliferation, the level of *E. coli* O157:H7 after 48 h of sprouting may have exceeded 4 CFU/g; therefore, it gives a false estimate of assay sensitivity to state the seed inoculation level but not the level of pathogen in the spent irrigation water tested. Failure to state the level of pathogen present in the spent irrigation water samples collected for testing also makes it impossible to determine the sensitivity of the TaqMan PCR assay and the Quix *E. coli* O157 sprout assay (62).

In only a few reports were these experimental methods directly compared with the FDA-recommended rapid immunoassays or with official BAM methods (47). Stewart et al. (160) compared the capabilities to detect *E. coli* O157:H7 of the two FDA-recommended rapid immunoassays and the following methods: direct plating of enrichment preparations onto tellurite-cefixime-sorbitol MacConkey (TC-SMAC) and BCM O157:H7(+) agars; membrane filtration of spent irrigation water and incubation of the filters on BCM O157:H7(+) agar; and Ab-DEFT analysis of sprouts and spent irrigation water. Results were similar for all methods. In one study, the nucleic acid-based BAX PCR assay for *Salmonella* was equivalent in sensitivity to culture methods for detection of *Salmonella* inoculated onto alfalfa sprouts and reduced the overall testing period by a minimum of 2 days (152).

However, this study indicated that both the BAX PCR assay for *E. coli* O157:H7 and the FDA BAM culture methods were unable to consistently detect *E. coli* O157:H7 from inoculated alfalfa sprouts, presumably due to competition by native microflora during preenrichment or release of toxic plant components. The very high level of native microflora on sprouts was also suggested to be responsible for the relatively low detection sensitivity for *Salmonella, E. coli* O157:H7, and *Shigella* spp. compared to the detection sensitivity with other types of produce subjected to a multiplex PCR assay after nonselective enrichment (98). Another study indicated that the GENE-TRAK *Salmonella* DLP, the Assurance Gold for *Salmonella,* and the FDA BAM methods gave comparable results for detecting *Salmonella* from either naturally contaminated or inoculated sprouts and spent irrigation water but the BAX PCR assay for *Salmonella* was not as sensitive (162).

Further improvement of recovery of pathogens from sprouts may be achieved by the use of optimal processing methods and nonselective enrichment broths, by the use of immunomagnetic capture, and by modification of selective agar media. When three methods (washing, stomaching, and homogenizing in a rotor-stator homogenizer) were compared for processing inoculated alfalfa sprouts, stomaching resulted in slightly higher recoveries than the other two methods (17). Enrichment in buffered peptone water containing 0.5% sodium thioglycolate led to better recovery of unstarved and starved cells of EC O157 from inoculated radish sprouts than did enrichment in modified EC broth plus novobiocin or buffered peptone water alone (144). The use of modified EC broth plus novobiocin was also found to be detrimental for recovery of injured *E. coli* O157:H7 inoculated onto radish sprouts (76). Concentration of *E. coli* O157:H7 and *E. coli* O26 from enrichment broth by immunomagnetic separation techniques enhanced the sensitivity and specificity of detection (75, 77, 119, 183, 189). Improved differentiation of *E. coli* O157 from bacteria naturally present on radish sprouts was obtained by the addition of salicin and 4-methylumbelliferyl–β-galactopyranoside to the selective agar medium TC-SMAC (66).

Methods have also been devised for the isolation of parasites from sprouts. Mung bean sprouts were reported to have a higher occurrence of contamination with *Cryptosporidium* and *Giardia* than other types of produce in Norway (140). Oocysts of *Cryptosporidium* and *Cyclospora* spp. as well as cysts of *Giardia* were detectable on artificially inoculated mung bean sprouts by a combination of washing, sonication, and immunomagnetic separation (139, 141).

Testing each production batch for pathogens according to FDA recommendations is arguably the single most effective way to enhance the microbiological safety of sprouts. However, microbial testing during sprout production

has not been fully implemented by the industry. In a survey of California sprout growers to determine industry compliance with the FDA guidance (170), 67% of the growers surveyed indicated that testing of spent irrigation water according to the FDA guidance was performed, even though 94% of the firms surveyed had read and reviewed the guidance. The noncompliance may be due in part to the extra cost required for the testing. Some producers have proposed pooling samples from multiple batches to lower the testing cost. The FDA is concerned that pooling will reduce the sensitivity of tests due to the dilution of the level of pathogen in a contaminated sample with samples that are not contaminated (157). One way to alleviate this concern is to institute a sample preconcentration step that would allow the entire pooled sample to be analyzed by a single test. For example, a 10-liter pooled sample of spent irrigation water from 10 production batches (1 liter from each batch) would be concentrated to an appropriate volume for analysis. A tangential flow filtration system has been developed that would allow the concentration of 10 liters of spent irrigation water into 100 ml within 2 h (64). Since sample concentration also results in an increase in the level of background microflora and other potentially interfering substances, the efficacy of the recommended rapid tests for detection of *E. coli* O157:H7 and *Salmonella* in the concentrated spent irrigation water was evaluated. It was found that the Assurance Gold enzyme immunoassay and the Reveal assay were able to detect the presence of 1 CFU of *Salmonella* and *E. coli* O157:H7 in 10 ml of concentrated sample (which corresponds to 1 liter of sprout water sample prior to filtration) (T. Fu, unpublished results). These results suggested that, with the addition of a sample preconcentration step, the entire pooled sample may be analyzed with a single test while the sensitivity of the test is maintained.

Pathogen Sampling and Detection in Seeds

There is a need to develop methods for seed testing, especially when large numbers of samples need to be tested quickly, for example, in conducting trace backs in outbreak investigations. The isolation of bacterial pathogens from seeds has been a subject of several research efforts.

Contamination of seeds by pathogens appears to be nonhomogeneous and to take place at low levels, and recovery is difficult. Despite the fact that *Salmonella* and *E. coli* O157:H7 have been shown to survive on artificially and naturally contaminated seeds for up to 2 years or more depending on the storage conditions (11, 68, 90, 95, 100, 166, 189), pathogens often were not isolated from the seed lots implicated in illness outbreaks (14, 15, 117). Nevertheless, when successful, seed testing has played an important role in outbreak investigations (84, 85).

Although there is some evidence that individual seeds in naturally contaminated lots may harbor pathogen populations of greater than 4 $\log_{10}$ CFU (169), other studies have estimated populations of *Salmonella* in naturally contaminated seed lots to range from 0.07 most probable number (MPN)/100 g to approximately 10 MPN/100 g (1, 84, 85, 100). With the often low overall contamination level, a sample size larger than the standard 25 g is needed for testing of outbreak-associated seeds (164). Inadequate sampling and small sample sizes have been suggested as the reasons for the failure of laboratory analyses in detecting *E. coli* O157:H7 and *Salmonella* in implicated seed lots (16). Testing of sprouting seed may increase the chance of finding target pathogens. Evidence exists that salmonellae can be cultured from naturally contaminated seeds only after sprouting (130).

Inami et al. (85) compared two processing methods for recovery of *Salmonella* from naturally contaminated alfalfa seeds. Seeds were either sprouted or shredded in a blender before preenrichment and culture. Results indicated that the two methods were comparable for detection of the pathogen. Liao and Fett (100) reported that two sequential preenrichment steps in buffered peptone water, rather than a single preenrichment step, prior to selective enrichment led to a higher detection rate for *Salmonella* from naturally contaminated seeds, indicating the possible presence of injured bacterial cells. Suslow et al. (164) found that the sensitivity of detection of *S. enterica* serovar Mbandaka from seeds associated with a multistate salmonellosis outbreak was improved by a combination of nonselective and selective enrichment, followed by immunocapture and plating or immunocapture in combination with PCR. Liao and Shollenberger (99) found that of five indicator agar media compared for the detection of *Salmonella* in the presence of native seed microflora, modified semisolid Rappaport-Vassiliadis medium was the most sensitive. Wu et al. (189) reported that the optimal procedure for isolation of *E. coli* O157:H7 from artificially inoculated alfalfa seeds was to first soak seeds for 1 h in sterile water, pummel for 1 min, enrich in modified tryptic soy broth, concentrate the cells by immunocapture, and plate onto selective agar media (Table 3). The use of immunomagnetic beads was also recommended by Liao and Shollenberger (99) to eliminate substances in seed homogenates that are inhibitory of PCR (Table 3). By combining immunomagnetic separation with the BAX PCR system for screening *Salmonella*, as few as 2 to 5 CFU of heat-injured *Salmonella* in 25 g could be detected within 24 h. The parasites *Cryptosporidium* and *Giardia* have also been isolated from naturally contaminated seeds, and on the basis of research results, testing of seeds rather than spent irrigation water was recommended for detection of these two parasites (142).

Seed screening has been proposed as an additional step in a multiple-hurdle approach to prevent sprout-associated outbreaks (143). Proper sampling, inspection, and testing of seeds for pathogens can substantially reduce the chance of using contaminated seeds for sprout production and therefore help prevent foodborne illness. However, improper seed screening protocols may still result in outbreaks (71).

A comprehensive seed-sampling and testing procedure has been developed by the sprouting industry (87). The procedure consists of a six-step process: sampling of seeds in bags that pass initial inspection (e.g., no evidence of rodent urine or bird droppings, and no holes in the bags), seed inspection under magnification, sprouting of seeds, spent water sampling, enrichment of sampled water, and pathogen testing. Briefly, the procedure calls for a sampling of at least 3 kg of seeds from each seed lot with 25-g subsamples from each bag. Assuming the level of pathogens found in seeds to be as low as 4 CFU per kg (117) and assuming uniform distribution of pathogens in seeds, a seed sample of 3 kg would give a probability of 99.9994% of finding a single pathogen cell in a 20-ton lot of seed (87). The entire sample is sprouted without prior sanitization by using commercial sprout production methods in an area segregated from commercial sprout production and at temperatures that support maximal pathogen growth if any pathogens are present. At 48 h of sprouting, a sample of the runoff water is collected and tested for pathogens by using FDA-recommended procedures (49). The adaptation of these seed-screening procedures has already prevented at least four potential sprout-related outbreaks due to *Salmonella* and *E. coli* O157:H7 (86).

REDUCING THE RISK OF FOODBORNE ILLNESS

Because of the exponential growth of microorganisms that occurs as a normal part of sprouting, measures must be taken to prohibit contamination both before and during production. It is generally believed that most of the outbreaks linked to sprouts have been due to the use of contaminated seeds. However, pathogens may enter the process via water, equipment, or handling and may present a hazard even if uncontaminated seeds are used. Thus, it is recognized that the risk of pathogen entry will be reduced by having in place not only good agricultural practices (GAPs) for seed production but also good manufacturing practices (GMPs) in the sprouting facility. Although sprout growers may be able to implement GMPs within their own facilities, adherence to the primary aspects of GAPs in producing seeds for sprouting is generally not within their control. Therefore, the potential presence of pathogens must be assumed, and sprout growers should also follow a pro-

gram of seed disinfection and pathogen testing during production to enhance the safety of their product. The food safety challenge presented by the unique nature of sprouts requires a system of complementary risk reduction measures, i.e., an integrated pathogen management approach.

GAPs

Seeds that are commonly used for sprouting are generally not intended for human consumption. Alfalfa seeds, for example, are produced primarily to sow a pasture or cover crop. They have typical characteristics of a raw agricultural product, including exposure to fecal contamination from wild and domestic animals, manure, and soil. Their use for human food purposes is merely incidental to their primary function in the agricultural sector. Nevertheless, seeds for sprouting are transformed into human food in a very brief process, and so it is FDA policy to regulate the seeds themselves as food; i.e., the seeds are not exempt from sanitation inspections and seizure and condemnation if they are adulterated (46).

The FDA has issued recommendations for the production and distribution of seeds for sprouting (49) which call for following the GAPs that are pertinent to the growing of fresh fruits and vegetables (48). The purpose of these comprehensive measures is to minimize the likelihood of contamination by pathogens, and they are to be tailored for specific growing, harvesting, and packing procedures as appropriate for the various crops. Among the recommendations that apply to seeds for sprouting are reduction of microbial hazards from the use of manures and biosolids from municipal waste treatment, consideration of water sanitary quality, attention to worker hygiene, and pest control in packing and distribution. Contaminated seed conditioning (cleaning) equipment can lead to cross-contamination of seed lots. Such equipment should be thoroughly cleaned and sanitized between seed lots destined for sprouting for human consumption.

Even if the seeds have been produced under GAPs, mixing with other lots during seed distribution or sprouting may occur. The ability to conduct trace backs in the event of an illness outbreak may limit the number of illnesses by allowing regulators to identify seed lots that have been implicated in the outbreak. Therefore, trace back is recognized as an important risk reduction strategy and complement to GAPs. Trace back was elevated from recommended to mandatory with the passage of the Public Health Security and Bioterrorism Preparedness and Response Act of 2002 (57), which requires food facilities to establish and maintain records of the immediate sources and immediate recipients of food (i.e., "one up, one down"). Seed distributors and sprout growers are required to maintain these records.

GMPs, Seed Treatment, and Pathogen Testing

The food industry has long been required to follow GMPs in manufacturing, packaging, or holding food, and the FDA reminded the sprout industry of this commitment in its guidance documents (49). Current GMPs include sanitary operation, worker hygiene, maintenance of facilities and equipment so as to protect against contamination, and control in production, processing, warehousing, and distribution (56).

In addition to GMPs, the FDA guidance documents made two further recommendations: (i) the use of an approved treatment for reducing pathogens on the seeds immediately before sprouting and (ii) testing of each production batch for *Salmonella* and *E. coli* O157:H7. The preferred test material is the spent irrigation water, i.e., the water that has flowed over and through the sprouts during production. If collection of the water is impractical due to methodology or equipment constraints, then the sprouts themselves may be tested. Such end-product testing for pathogens is generally considered to be an ineffective safety assurance technique for most foods because of the low levels and sporadic nature of contamination. Seed sprouting, however, uniquely provides enrichment conditions leading to high population levels of the microbial flora; thus, sampling problems associated with sporadic, low-level contamination are less likely to occur. In fact, for sprouting, pathogen testing is an extremely effective technique and one proven by the sprout industry as a means to avoid distribution of contaminated product (86). A recent Monte Carlo simulation model using the available literature predicted that sprout sampling and spent irrigation water sampling would be more effective in the detection of pathogens than seed sampling prior to production (113).

HACCP Systems and Integrated Pathogen Management

Traditionally, hazard analysis and critical control point (HACCP) systems provide one or more critical control points in a food manufacturing process that ensure safety of the product. Although this key element is missing for sprout production (no treatment has been shown to effectively control pathogen growth), sprout growers have adopted elements of HACCP systems to enhance safety (88). Retail establishments that grow sprouts for direct sale to consumers must employ a HACCP plan according to the FDA Food Code (51, 58).

Sprout growers have advocated an integrated risk reduction approach involving not only seed sanitization and pathogen testing of spent irrigation water from production batches but also a seed certification and screening program. Acceptability of seeds prior to production sprouting would be determined by inspecting the seed shipment for evidence of filth and sam-

pling the seeds according to a statistically based sampling plan. A composite seed sample is sprouted, and pathogen testing of the spent irrigation water by FDA-recommended procedures is performed. This seed-screening procedure does not preclude the necessity of testing each production batch according to the FDA guidance. All of these measures, applied together, are the best available options for reducing the microbiological risks associated with sprouts.

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